

## **Solid Phase Extraction of Cocaine and Metabolites from Biological Specimens**

### **1 Introduction**

Cocaine is a naturally occurring stimulant that is found in the leaves of the *Erythroxylon coca* plant. The primary metabolites of cocaine in humans are benzoylecgonine and methylecgonine. Cocaethylene is another biotransformation product of cocaine that is produced when cocaine and ethanol are used together.

### **2 Scope**

This procedure is used to confirm and quantitate cocaine (COC), benzoylecgonine (BE) and cocaethylene (CE) in biological specimens; it is also used to identify methylecgonine (EME), but results are not reported quantitatively. It is derived from "Cocaine and Benzoylecgonine in Serum, Plasma, or Whole Blood" which is published in the Clean Screen Application Manual by Worldwide Monitoring. The published extraction procedure of biofluids is essentially followed intact, but analysis is accomplished by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI -MS/MS).

### **3 Principle**

Biological specimens are assayed for the presence of cocaine and metabolites. Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. The specimens are prepared for solid phase extraction (SPE) via centrifugation and/or dilution. Extractions are accomplished through the use of Clean Screen DAU SPE cartridges. Cocaine and metabolites are eluted from the SPE cartridge using a mixed solvent system of methylene chloride, isopropanol, and ammonium hydroxide. The eluent is taken to dryness, reconstituted in unbuffered LC mobile phase and analyzed directly by LC-ESI-MS.

### **4 Specimens**

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate (1:1 in deionized water). Typically, 1 mL of specimen is used for a screen or for qualitative analysis. For quantitative analysis, one – 1.0 mL aliquot and one – 0.5 mL aliquot will be analyzed. In instances where sample volume is limited or there is reason to suspect a sample of being a strong positive, (due to case history or a saturated immunoassay response) a smaller volume of specimen, diluted to 1.0 mL with deionized water, may be used.

## 5 Equipment/Materials/Reagents

- a. 12 x 75 mm test tubes
- b. 16 x 100 mm test tubes
- c. Vortexer
- d. Centrifuge
- e. Clean Screen DAU SPE Cartridges (United Chemical Technologies, Bristol, PA)
- f. Solid phase extraction vacuum manifold or positive pressure manifold
- g. Liquid Chromatograph-Mass Spectrometer capable of data dependent tandem operation in the electrospray ionization mode
- h. Grace 5  $\mu$ m particle silica HPLC column, 2.1 x 150 mm (or equivalent)
- i. Miscellaneous routine laboratory glassware and supplies
- j. Deionized water
- k. Methanol (HPLC grade)
- l. Acetonitrile (HPLC grade)
- m. 0.1 M Sodium Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify  $5.8 < \text{pH} < 6.1$ . Store refrigerated in glass. Stable 2 months.
- n. SPE Elution Solvent (78:20:2 Methylene Chloride:Isopropanol:Ammonia):  
Combine 20 mL HPLC grade isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL HPLC grade methylene chloride and mix well. Store in glass at room temperature. Prepare fresh.
- o. 0.1 M Hydrochloric Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well. Store in glass at room temperature. Stable 6 months.
- p. 95:5 Methanol:Water:  
Combine 95 mL methanol (HPLC grade) and 5 mL deionized water in a graduated cylinder.

Mix well. Store in glass or plastic at room temperature. Stable 12 months.

- q. LC Mobile Phase (95:5:0.03 Methanol:Water:Ammonia):  
Combine 950 mL HPLC grade methanol and 50 mL deionized water. Mix well. Add 0.3 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 2 weeks.
- r. pH paper
- s. Evaporator with nitrogen
- t. Rotator

## 6 Standards and Controls

- a. d<sub>3</sub>-Cocaine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- b. d<sub>3</sub>-Methylecgonine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- c. d<sub>3</sub>-Cocaethylene Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- d. d<sub>8</sub>-Benzoylecgonine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- e. Internal Standard Working Mixture (d<sub>3</sub>-Cocaine, d<sub>3</sub>-Methylecgonine, d<sub>3</sub>-Cocaethylene, and d<sub>8</sub>-Benzoylecgonine - 10 µg/mL):  
To a 10-mL volumetric flask, add 5 mL of acetonitrile and 1 mL each of the d<sub>3</sub>-cocaine, d<sub>3</sub>-methylecgonine, d<sub>3</sub>-cocaethylene and d<sub>8</sub>-benzoylecgonine stock standards. Add 20 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least 2 years.
- f. Cocaine Stock Standard (1.0 mg/mL):  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- g. Methylecgonine Stock Standard (1.0 mg/mL):

Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.

- h. **Cocaethylene Stock Standard (1.0 mg/mL):**  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- i. **Benzoyllecgonine Stock Standard (1.0 mg/mL):**  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- j. **Intermediate Calibration Standard (Cocaine and Benzoyllecgonine - 20 µg/mL; Cocaethylene - 10 µg/mL):**  
To a 10-mL volumetric flask, add 200 µL each of the Cocaine and Benzoyllecgonine Stock Standards, and 100 µL of the Cocaethylene Stock Standard. Add 50 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least one year.
- k. **Working Calibration Standard (Cocaine and Benzoyllecgonine - 2 µg/mL; Cocaethylene - 1 µg/mL):**  
Add 1.0 mL of the Intermediate Calibration Standard to a 10-mL volumetric flask. Add 50 µL of 0.1 M HCl. Dilute to the mark with deionized water. Mix well. Prepare fresh.

Table 1 shows the concentrations and volumes used for preparation of typical calibrators.

Table 1: Typical Calibrator Preparation for Quantitations

Calibrator Level (ng/mL)	Volume of Matrix (mL)	Volume of Working Calibration Standard (mL)
0	1.0	0
50/25	1.0	0.025
100/50	1.0	0.050
400/200	1.0	0.200
700/350	1.0	0.350
1000/500	1.0	0.500

- l. **Intermediate Control Standard (Cocaine and Benzoyllecgonine - 80 µg/mL; Cocaethylene and Methylecgonine - 40 µg/mL):**  
To a 10-mL volumetric flask, add 800 µL each of the Cocaine and Benzoyllecgonine Stock Standards, and 400 µL each of the Cocaethylene and Methylecgonine Stock Standards. Add 50 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least one year.

- m. Working Control Standard (Cocaine and Benzoyllecgonine – 8 µg/mL; Cocaethylene and Methylecgonine – 4 µg/mL):  
 Add 1.0 mL of the Intermediate Calibration Standard to a 10-mL volumetric flask. Add 50 µL of 0.1 M HCl. Dilute to the mark with deionized water. Mix well. Prepare fresh.
- n. Positive Control:  
 Prepared in-house on the day of extraction as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Typical control concentrations are 160 and 800 ng/mL for benzoyllecgonine and cocaine and 80 and 400 ng/mL for cocaethylene and methylecgonine. A Positive Control is extracted and analyzed with every quantitative assay. The Positive Control will be matrix matched, when possible. (The deuterated analogs also serve as qualitative positive controls for each specimen.)
- Typical Positive Control Preparation:
- |              | µL Working Control Standard to add to 1 mL blood |
|--------------|--|
| Low Control  | 20   |
| High Control | 100  |
- o. Negative Control:  
 Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Clinical Controls International, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control is extracted and analyzed with every quantitative assay. The Negative Control will be matrix matched, when possible.
- p. LC/MS Performance Standard (1 µg/mL each of cocaine, benzoyllecgonine, cocaethylene, and methylecgonine):  
 To a 25 ml volumetric flask, add 25 µl each of the benzoyllecgonine, cocaethylene, cocaine, and methylecgonine stock standards. Fill to the mark with acetonitrile. Store in glass below 0°C. Stable for at least one year. A 5 µl portion of this mixture is analyzed by LC/MS/MS under the instrumental conditions given in Section 10 of this procedure each day before the instrument is used for case samples.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 100 mm test tubes add 1 mL of control or biological fluid or 1 g of prepared tissue homogenate.
- b. Prepare calibration curves as shown in Table 1 above.
- c. Add 25  $\mu$ L of the Internal Standard Working Mixture. This will result in working concentrations of 250 ng/mL of each deuterated analog.
- d. Bring all samples to approximately 5 mL with deionized water and vortex.
- e. Whole blood and tissue homogenates (skip to step f. for urine, serum, or vitreous specimens) should stand for 5 minutes before centrifuging for 10 minutes. Discard any resulting pellet.
- f. Add 2 mL of 0.1 M phosphate buffer to each specimen. Vortex.
- g. Verify pH of each specimen is  $6.0 \pm 0.5$ .
- h. Prepare SPE cartridges.
- i. Pre-rinse SPE extraction cartridge by adding 3 mL of Elution Solvent followed by 3 mL of methanol.
- j. Condition column with 3 mL of deionized water followed by 1 mL of 0.1 M phosphate buffer.
- k. Load sample on SPE cartridge.
- l. Rinse column with 2 mL of deionized water, 2 mL of 0.1 M hydrochloric acid, and 3 mL of methanol.
- m. Dry column under full vacuum for 90 seconds.
- n. Apply 3 mL of Elution Solvent and collect eluent in 12 x 75 mm test tubes.
- o. Evaporate to dryness under nitrogen at 40°C.
- p. Reconstitute the residue in 100  $\mu$ L of 95:5 methanol:water.

- q. Prior to analysis of any case samples by LC/MS/MS, analyze 5 µL of the LC/MS Performance Standard using the instrumental parameters that follow in order to verify that the system is working properly. If the decision criteria in Section 11.1 of this procedure are met, proceed to step r. Otherwise, perform any appropriate instrument maintenance.
- r. Inject 5 µL of the extract into the LC/MS operated in positive ion electrospray ionization mode.

## 9 Instrumental Conditions

Following are the operating parameters for the instruments used in this procedure. Appendix 2 contains an abbreviated version of instrumental parameters used in this procedure that may be used by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Parameters		Column Parameters	
composition	95:5:0.03 methanol: water: ammonia	type	silica
isocratic flow	0.3 mL/min	length	150 mm
run time	15 min	internal diameter	2.1 mm
temperature	ambient	particle size	5 µm
		column temp	30°C

## 9.2 Mass Spectrometer Parameters

Segment #1 (0 - 5 min)			
Scan Event #1		Scan Event#2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan; unit resolution	scan mode	product ion MS/MS; unit resolution
scan range	260 - 360 m/z	precursor ions	290, 304 and 318 m/z
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		collision energy	45% relative
		isolation width	1.5 AMU
		product scan range	software control
Segment #2 (5 – 15 min)			
Scan Event #1		Scan Event#2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan; unit resolution	scan mode	product ion MS/MS; unit resolution
scan range	170 - 230 m/z	precursor ion	200 m/z
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		collision energy	40% relative
		isolation width	1.5 AMU
		product scan range	70 - 230 m/z

## 10 Decision Criteria

### 10.1 LC/MS Performance Standard Decision Criteria

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should generate Gaussian shaped chromatographic peaks. The following molecular ions should be traced for each analyte: cocaine – 304, methylecgonine – 200, cocaethylene – 318, benzoylecgonine – 290.

The retention times of the 4 analytes should be within  $\pm 5\%$  of the previous run of the performance standard. Minor changes in mobile phase percentage may account for slight retention time shifts.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

### **10.1.2 Mass Spectrometry**

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the four analytes in the performance standard should be present. The following molecular ions should be present as the base peak for each analyte: cocaine – 304, methylecgonine – 200, cocaethylene – 318, benzoylecgonine – 290.

### **10.2 Batch Acceptance Criteria**

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All analytes should be detected in the Positive Control. Each Quantitative Positive Control should quantitate within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

### **10.3 Analyte Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, the criteria in sections 11.2.1 through 11.2.3 should be met in order to identify cocaine, benzoylecgonine, methylecgonine or cocaethylene within a biological specimen:

#### **10.3.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

##### **10.3.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

##### **10.3.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

### 10.3.2 Analyte Stability

In order to ensure that there has been no significant hydrolysis of cocaine during the sample preparation and analysis, ensure that any peak for d<sub>3</sub>-benzoylecgonine (trace m/z 293 in full scan) is less than 1% of the area for d<sub>8</sub>-benzoylecgonine (trace of m/z 298). If this criterion is not met, results may still be used for qualitative identification of the various analytes of interest, but no quantitative results from the assay should be reported.

Note: If the d<sub>8</sub>-benzoylecgonine has appreciable amounts of d<sub>3</sub>-benzoylecgonine in it (which happens in an occasional lot; the user will know this is the case if all samples in a batch fail the stability test), further steps may be necessary.

1. With the batch, analyze a portion of the 10 ppm Internal Standard working solution 10-fold diluted in 95/5 methanol water.
2. Measure the d<sub>3</sub>-benzoylecgonine to d<sub>8</sub>-benzoylecgonine ratio in this sample.
3. The d<sub>3</sub>-benzoylecgonine to d<sub>8</sub>-benzoylecgonine ratio in the extracted samples may not exceed this ratio by more than 0.01 (1% absolute).

### 10.3.3 Mass Spectrometry

The MS/MS fragmentation spectra should meet the following independent criteria for each compound identified.

- a. Cocaine: (fragments of m/z 304) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.
- b. Cocaethylene: (fragments of m/z 318) The base peak should be m/z 196, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.
- c. Benzoylecgonine: (fragments of m/z 290) The base peak should be m/z 168, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 150 and m/z 272.
- d. Methylecgonine: (fragments of m/z 200) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 82 and m/z 156.

### 10.4 Reporting Cocaine

To report cocaine qualitatively based upon this method, the area of the M+H peak for cocaine must be greater than or equal to 5% of the M+1 peak for benzoylecgonine. Quantitative results for cocaine that are 5% or less than the benzoylecgonine amount measured may be reported if the

results for cocaine are above 50 ng/mL.

## 11 Calculations

Linear regression analysis with 1/x weighting is performed for cocaine, benzoylecgonine and cocaethylene quantitation. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices for calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- a. Linearity: Cocaine: 50 - 1000 ng/mL  
 Benzoylecgonine: 50 - 1000 ng/mL  
 Cocaethylene: 25 - 500 ng/mL
- b. Limit of Detection: Cocaine: 10 ng/mL, or lower  
 Benzoylecgonine: 10 ng/mL, or lower  
 Methylecgonine: 5 ng/mL, or lower  
 Cocaethylene: 5 ng/mL, or lower

- c. Bias:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	3.07	-0.32	-1.00
BE	-1.41	-2.52	-3.22
CE	15.61	7.95	5.11

d. Repeatability:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	4.63	3.25	5.14
BE	2.50	3.23	2.35
CE	5.81	4.35	4.32

e. Intermediate Precision:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	8.46	3.33	5.63
BE	4.31	4.11	2.40
CE	6.71	5.46	6.73

f. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Solid Phase Extraction Application Manual*; United Chemical Technologies. Lewistown, PA, 2004; pp. 29-31.

Mule, S. J.; Casella, G. A. *J. Anal. Toxicol.* 1988, 12, 153-155.

Jeanville, P.M.; Estape, E.S.; et al. *J. Am. Soc. Mass Spectrom.* 2000, 11, 257-263.

Jeanville, P.M.; Estape, E.S.; et al. *J. Anal. Toxicol.* 2001, 25, 69-75.

*FBI Laboratory Safety Manual*.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

FBI Laboratory Chemistry Unit - Instrument Operation and Support Subunit SOP Manual.

Rev. #	Issue Date	History
4	02/27/13	In 5h, updated column brand name. Added cocaine reporting criteria in 11.3.
5	10/01/14	Added instructions for preparation of reagents in Section 5. Added a second source for standards in Sections 6f, 6h and 6i. Updated calibration and control preparation instructions in Section 6j – 6n. Moved Table 1 to Section 6k, removed Calibration Section (7) and renumbered subsequent sections. In Section 8d, updated step involving addition of water to samples for clarity. Removed reference to high flow cartridges from 8h. Added Section 10.2 and renumbered subsequent sections. Added text to 10.3.2 to explain what to do if the d <sub>3</sub> -benzoylecgonine standard is contaminated with d <sub>3</sub> -benzoylecgonine. Removed reagent preparation instructions from Appendix 1. Reformatted Appendix 2 to include all pertinent instrumental parameters.
6	09/28/15	Removed references to Tox 103 (reagent SOP) in Sections 5 and 15. In Section 5.q, updated mobile phase expiration to 2 weeks. Removed methylecgonine from 6.j and 6.k since it is not quantitated. Updated worksheet in Appendix 1.

**Approval**

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**Appendix 1: Abbreviated version of the SPE Cocaine Procedure for bench use.**

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**Appendix 2: Abbreviated instrumental parameters for the SPE Cocaine Procedure for bench use.**

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